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Good Afternoon,
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Song-Ping Liang et al., "Covalent Immobilization of proteins and peptides for solid phase sequencing using prepacked capillary columns"
Analytical Biochemistry 188, (1990) pp. 366-373.

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ANALYTICAL BIOCHEMISTRY 188, 366–373 (1990)

Covalent Immobilization of Proteins and Peptides for Solid-Phase Sequencing Using Prepacked Capillary Columns

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The use of prepacked capillary columns for immobilizing proteins and peptides for solid-phase Edman degradation is described. Capillary tubes with an internal volume of about 30 μl are filled with glass beads bearing isothiocyanato groups (DITC-glass), aminophenyl groups (AP-glass), or aminoethylaminopropyl groups (AEAP-glass) and are sealed with porous plugs. Proteins or peptides in appropriate buffers are introduced into the columns by capillary action and are covalently coupled to the glass beads, either by reaction of lysine side-chain amino groups with DITC-glass, by carbodiimide-mediated reaction of carboxyl groups with AP-glass, or by reaction of homoserine lactone groups with AEAP-glass. Optimization of attachment conditions is described. The capillary columns are loaded into the sequencer and, when sequencing has been completed, are discarded. This technique greatly simplifies polypeptide immobilization and is suitable for microsequencing (<50–1000 pmol) or macrosequencing (1–50 nmol).

The success of the solid-phase version of the Edman degradation (1) depends on the immobilization of peptides and proteins by covalent attachment to an insoluble support matrix. The advantage of covalent immobilization is that mechanical losses of sample are avoided, a factor likely to become of greater significance for sequencing of very small (subpicomole-level) samples (2). Although methods have been developed over the years that permit immobilization of almost any polypeptide (3–5), some investigators have found existing techniques unreliable or inconvenient. Furthermore, present-day demands for picomole-level sequencing require changes of scale, e.g., miniaturization of reaction cells and other components, to reduce contaminants.

Recently, a second-generation solid-phase sequencer, the MilliGen/Biosearch ProSequencer, which is designed for sequence analysis at the 100-pmol or lower level, has been described (6). This instrument will handle proteins and peptides immobilized on polyvinylidene difluoride (PVDF)[2] membranes (7) or on glass beads. Because glass bead supports have a number of convenient characteristics (high capacity, physical and chemical stability) and have been used for a number of years, we have sought to improve glass bead immobilization technology. The prepacked, disposable capillary columns we describe here greatly simplify polypeptide immobilization and, by reducing handling steps, remove some of the sources of variable yields.

METHODS

Materials. Underivatized controlled-pore glass (CPG) beads (170 Å pore size, 200–400 mesh) were purchased from Sigma or from CPG Inc. (Fairfield, NJ), and derivatized CPG beads were either prepared as described below or purchased from CPG Inc. *N*-(2-Aminoethyl)-3-aminopropyltrimethoxysilane and 3-aminopropyltriethoxysilane were obtained from Pierce Chemical; 1,4-phenylene diisothiocyanate (DITC), from Eastman; 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 4-nitrobenzoyl chloride from Aldrich.

Preparation of aminopropyl-glass and aminoethylaminopropyl-glass. Controlled-pore glass beads were silylated by a modification of the procedure of Wachter *et al.*

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[2] Abbreviations used: PVDF, polyvinylidene difluoride; CPG, controlled-pore glass; DITC, 1,4-phenylene diisothiocyanate; AP, aminophenyl; AEAP, aminoethylaminopropyl; DMF, dimethylformamide; SDS, sodium dodecyl sulfate; EDC, *N*-ethyl-*N'*-dimethylaminopropylcarbodiimide; PTH, phenylthiohydantoin.

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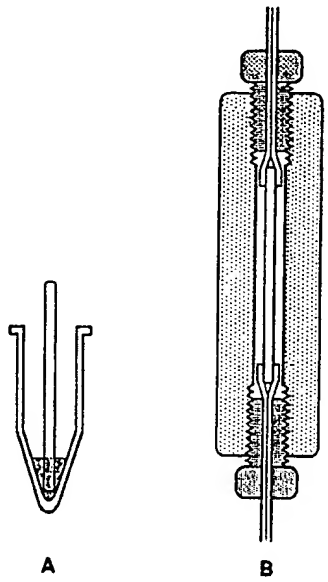

FIG. 1. Capillary glass bead column. During attachment reactions, the column is placed vertically in an Eppendorf microcentrifuge tube (A), and the sample fills the column by capillary action. For sequencing, the flared ends of two pieces of Teflon tubing are placed over the ends of the capillary, and the column is placed in an aluminum column holder (B) and is secured by means of two $\frac{1}{4}$-28 plastic tube end fittings.

(8). CPG beads (4 g, 170 Å, 200–400 mesh) were boiled for 10 min in 10 mM HCl and washed with 200 ml of distilled water, followed by 200 ml of methanol, and dried under vacuum for 2 h over P_2O_5. The beads were then heated for 2 h at 200°C and allowed to cool under vacuum over P_2O_5. A solution of 0.3 ml of 3-aminopropyltriethoxysilane [or 1.0 ml of *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane] in 30 ml of dry toluene was added, and then the beads were degassed under vacuum and heated at 56°C for 24 h with occasional stirring. The beads were filtered on a sintered glass filter, washed with HPLC-grade methanol, and dried under vacuum at room temperature over P_2O_5. The beads were stored at 4°C under nitrogen.

Preparation of aminophenyl-glass. AP-glass beads either were purchased from CPG Inc. or were prepared by suspending 1 g of AP-glass beads in 5 ml of DMF and 5 ml of triethylamine in a 20-ml vial and adding dropwise, while stirring, 600 mg of *p*-nitrobenzoyl chloride in 1 ml of DMF. The reaction mixture was stirred for 20 min at room temperature and then filtered on a sintered glass filter. The beads were washed alternately with pyridine, methanol, and water (2 × 50 ml of each) and were dried under vacuum. A 10-mg sample was removed to test for the presence of unreacted amino groups. At this point the acylation of the amino group should be 95–98% complete. The nitro group was reduced by suspending the nitroglass beads in 10 ml of DMF in a stoppered flask, heating to 70°C in a water bath, and adding 1.5 g of $SnCl_2$ in 2 ml of DMF (which had been warmed to 70°C) dropwise with stirring of the beads. The temperature was raised to 100°C and maintained for 20 min. Concentrated HCl (3 ml) was added and the stirred mixture was kept at 100°C for 15 min. The beads were filtered on a sintered glass filter and washed with 50 ml of concentrated HCl to remove tin salts, then alternately with water and methanol (2 × 50 ml each). The beads were dried under vacuum and stored at 4°C under nitrogen. The amino content of the beads was about 50 nmol/mg, as determined by the trinitrobenzenesulfonic acid assay (9).

Preparation of DITC-glass. Aminopropyl-glass beads (2 g) were suspended in 6 ml of dry tetrahydrofuran (freshly distilled from sodium and benzophenone) containing 200 mg of DITC. The mixture was kept at room temperature for 2 h under nitrogen (or argon), and was then filtered and washed with 100 ml of benzene (the filtrate and benzene washes can be concentrated to recover DITC, which is repurified by crystallization). The beads were finally washed with 150 ml of anhydrous methanol and were dried under vacuum. The beads were stored at 4°C under nitrogen (8).

Preparation of capillary columns with sintered glass plugs. A 100-μl disposable micropipet (Clay–Adams Accu-fill 90 micropet, No. 4625; other brands have walls that are too thin and fragile; the total volume of these pipets is about 120 μl) is cut into four small columns (1.3 mm i.d. × 32 mm; 30-μl volume). A small amount of ground Pyrex glass wool is pressed into one end to make a plug about 2 mm thick. The end of the tube is touched to a gas flame to sinter the glass wool and to fire-polish the end of the tube. The remainder of the tube is filled with DITC-glass or AP-glass (about 10 mg), and the other end of the tube is sealed with a sintered glass plug as described above. The columns are stored at 4°C.

Preparation of capillary columns with PVDF plugs. A 100-μl disposable micropipet is cut into four small columns (1.3 mm i.d. × 32 mm; 30-μl volume), and the ends are fire-polished carefully so as not to close off the tube. A solution of PVDF is made by adding 1 g of PVDF membrane (Millipore Corp.) in 15 ml of DMF and heating at 60°C for 15 min or until the PVDF dissolves completely. A number of tubes (up to 50 or more) are bundled together by means of a rubber band, and the bundle is tapped on a flat surface until all the tubes are even at one end. The bundle is placed upright on a Petri dish or beaker and CPG beads are sprinkled into the tubes so as to fill each tube to a depth of 2–3 mm. The tubes are tapped to compact the beads, and the bundle is carefully transferred to a beaker which has been filled to a depth of 3 mm with the PVDF solution. After the solution has entered the beads by capillary action, the bundle is carefully transferred to a beaker containing water to a depth

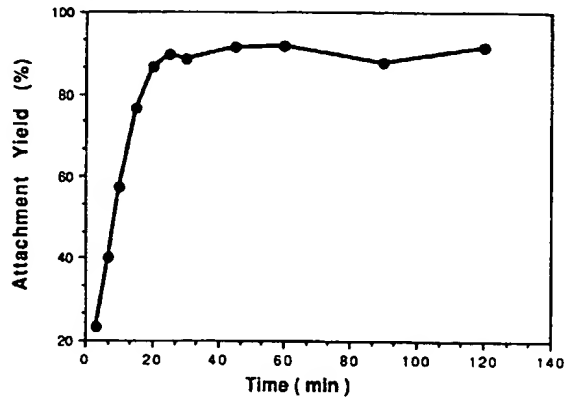

FIG. 2. Time course for attachment of β-lactoglobulin to DITC-glass beads in a capillary column. Protein samples (100 pmol each) of radioiodinated protein were coupled as described in the text. At intervals the reactions were stopped by washing unreacted protein from the column. Yields were calculated from the radioactivity of columns and washings.

of 5 mm. When the PVDF/DMF-impregnated beads contact the water, the DMF dissolves in the water and the PVDF precipitates, forming a porous plug in the end of the tube. After 5 min, the bundle is removed and the tubes are washed with methanol and dried in a vacuum desiccator. The bundle of tubes is then placed upright in a clean, dry beaker, and DITC-glass or AP-glass is added, with tapping, to fill the tubes to the top. When all of the tubes have been filled, approximately 2 μl of the PVDF solution is added to the top of each tube to wet the upper approximately 2 mm of the beads. The tubes are then inverted and placed in a beaker of water, as described above, to precipitate the PVDF. The tubes are then washed with methanol, blown dry with nitrogen, and further dried under vacuum to remove traces of solvent.

Attachment of polypeptides to DITC-glass in capillary columns. The peptide or protein sample (50–1000 pmol) is transferred to a 0.5-ml Eppendorf tube and dried in a Speed-Vac concentrator. (The sample should be free of salts and buffers containing primary or secondary amino groups.) The sample is redissolved in 15 μl of coupling buffer (0.2 M Na_2HPO_4, pH 9.0, 1% SDS), and one end of a capillary column containing DITC-glass is placed into the sample solution, which is drawn into the column by capillary action in about 10 s. An additional 15 μl of coupling buffer is added to the Eppendorf tube and it, too, is allowed to be drawn into the capillary column. Finally 15 μl more of buffer is added to the Eppendorf tube, and the tube and column are heated at 55°C for 45 min. The column is washed with coupling buffer (300 μl), water (300 μl), and methanol (600 μl) and is blown dry with nitrogen. In the case of labeled proteins

TABLE 1

Attachment and Sequencing Yields of Proteins and Peptides in DITC-Glass Capillary Columns[a]

Protein or peptide	Initial amount (pmol)	Attachment yield (%)	Sequenceable amount[b] (pmol)	Overall sequenceable yield[c] (%)
β-Lactoglobulin	100	88[d]	—	—
	100	89[e]	42	42
Myoglobin	100	80[d]	—	—
	100	90[e]	52	52
Glyceraldehyde-3-phosphate dehydrogenase	100	86[d]	—	—
Bovine serum albumin	100	92[d]	—	—
Trypsinogen	100	90[d]	—	—
Cytochrome c	100	83[e]	61	61
Ribonuclease a	100	85[e]	49	49
SAPYTYQSDLRYKL	80	—	38	48
WDYHNRMK	80	—	20	25
SHEGTFTSDYSKYLDSRRAQSFVQWL	110	—	39	36
CVEQLSPEEEEKRAIRRERNKAAA	58	—	22	38
MLRFVTK	54	—	27	50
ELYENKPRAPYIL	100	—	59	59

[a] The attachment was performed at 55°C for 30 min.

[b] The amount of attached peptide capable of being sequenced was determined by integrating the areas of the first three PTH peaks and extrapolating to zero cycles.

[c] The overall yield reflects both the attachment and sequenceable yields.

[d] Attachment yields for ^{125}I-labeled proteins. Yields were calculated based on the combined radioactivity of the columns and washings. Duplicate samples were run and gave yields within 5% of each other.

[e] Yield determined by amino acid analysis. Duplicate samples were prepared; one was used for amino acid analysis and the other for sequencing.

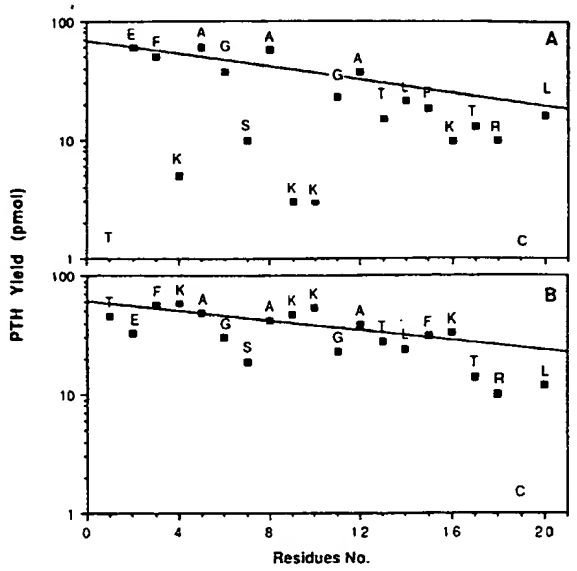

FIG. 3. Yields of PTH-amino acids on sequencing of samples of *S. cerevisiae* cytochrome c in capillary columns containing (A) DITC-glass and (B) AP-glass. Protein samples (100 pmol) were attached as described in the text and sequenced by solid-phase Edman degradation. Repetitive yields were calculated by comparing the yields of Ala-5 and Ala-12; these were 93.4 and 96.2% for DITC-glass and AP-glass, respectively. With DITC-glass, the N-terminal Thr, as well as most of the Lys residues, were attached to the support and were missing or detected in low yield. Cys-19 is conjugated to heme and was not detected.

where attachment yields are being monitored, the column is washed with coupling buffer (300 μl), 20% trifluoroacetic acid (300 μl), and water (600 μl), and the washings are analyzed for unbound peptide or protein.

Attachment of polypeptides to AP-glass in capillary columns. The peptide or protein sample (50–1000 pmol) is transferred to a 0.5-ml Eppendorf tube and dried in a Speed-Vac concentrator. (The sample should be free of salts and buffers containing carboxyl groups.) The sample is redissolved in 15 μl of coupling buffer (1.0 M pyridine·HCl, pH 5.0, 1% SDS), and the Eppendorf tube is placed in an ice bath. A solution of *N*-ethyl-*N'*-dimethylaminopropylcarbodiimide (EDC) in coupling buffer (1 mg in 5 μl) is prepared and immediately added to the sample. Immediately thereafter an ice-cold AP-glass capillary column is placed in the Eppendorf tube. After the sample solution has been drawn into the column (about 10 s), 15 μl of ice-cold coupling buffer is added to the Eppendorf tube to wash residual polypeptide into the capillary tube. The reaction is allowed to proceed for 15 min and the capillary tube is washed with 300 μl of 20% acetic acid to stop the reaction. The column is washed as described for DITC-glass.

Attachment of homoserine peptides to AEAP-glass in capillary columns. The peptide having a C-terminal homoserine (50–1000 pmol) is evaporated to dryness in a 0.5-ml Eppendorf tube in a Speed-Vac concentrator. The sample is redissolved in 100 μl of anhydrous trifluoroacetic acid, and the solution is kept at room temperature for 1 h. The solvent is evaporated in the Speed-Vac concentrator (which should be equipped with a solid NaOH trap), and the residual peptide is dissolved in 15 μl of coupling buffer (triethylamine:dimethylformamide: formamide, 1:3:4 v/v). One end of a capillary column containing AEAP-glass is placed in the sample solution, which is drawn into the tube in about 15 s. An additional 10 μl of coupling buffer is added to the tube, and it, too, is allowed to be drawn into the capillary. Finally, 15 μl more of coupling buffer is added to the Eppendorf tube and it and the column are incubated at 45°C for 2 h. The column is washed as described for DITC-glass.

Radiochemical methods. Test proteins were labeled with ^{125}I by the Iodobeads method (10). The radioactivity of proteins immobilized in capillary tubes was determined by counting the tubes before or after sequencing in a Beckman gamma counter.

Solid-phase Edman degradation. Peptides and proteins were sequenced by Edman degradation on prototypes of the MilliGen/Biosearch Model 6600 ProSequencer (6) using recommended reagents. Capillary columns containing immobilized polypeptides were placed in the column holder shown in Fig. 1B, which was placed in the heating block assembly of the sequencer. The column was then washed with methanol for 3 min, followed by coupling buffer for 1 min, before starting the run. Phenylthiohydantoins (PTHs) were collected after each cycle and analyzed on a Millipore/Waters Model 600 HPLC system equipped with a Model 712 WISP autoinjector.

RESULTS AND DISCUSSION

Our goals in developing the capillary column method were twofold: (1) to reduce the volume of reaction cell of the sequencer and (2) to find a simple way of avoiding

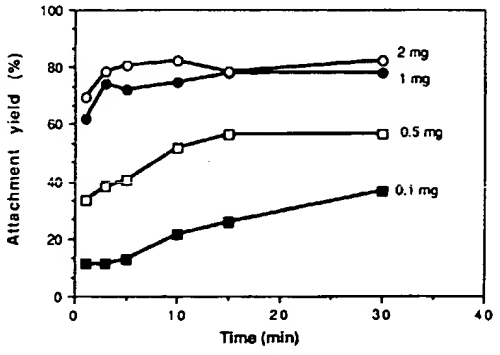

FIG. 4. Time course for attachment of β-lactoglobulin to AP-glass. Samples of 100 pmol of radioiodinated protein were coupled at room temperature as described in the text with differing amounts of EDC.

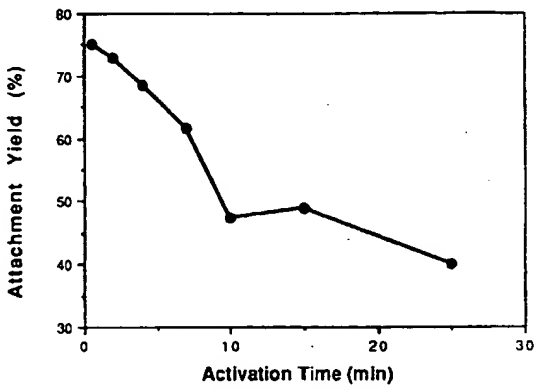

FIG. 5. Effect of incubation time after activation of proteins with EDC before attachment to AP-glass. Radioiodinated β-lactoglobulin was activated with EDC at room temperature and samples were removed at intervals for attachment to AP-glass.

sample handling problems encountered with earlier techniques (3). Reduction of the sample cell and other components is important in microsequence analysis, because it lowers the area of surfaces which can harbor adsorbed contaminants (11). In addition, small reaction cells significantly reduce the amounts of reagents consumed by the sequencer. Sample handling is a particular problem for persons unfamiliar with the chemistry of the attachment methods, and a more general problem for anyone faced with the difficulties of efficiently transferring small amounts (<10 mg) of glass beads from one container to another.

For many years, the method of choice for covalent immobilization of proteins has been attachment via lysine side-chain amino groups to DITC-glass (3,8). For peptides which do not contain lysine residues, carbodiimide-mediated coupling of peptide carboxyl groups to aminopolystyrene has proved effective (3,5). The mechanical and absorptive properties of aminopolystyrene are not suitable for microsequencing, however, and for this reason we have utilized in the present studies AP-glass, which combines the desirable properties (high surface area, rigidity) of glass beads with greater nucleophilicity of arylamines at pH 5, which is the optimal pH for carbodiimide coupling reactions. And finally, we have also adapted the capillary column technique for use with a β-diamine support, AEAP-glass, which can be used to couple homoserine peptides, generated by cyanogen bromide cleavage, at the C terminus (12).

The capillary column technique is adaptable to all three types of sequencing support. The column is constructed from a section of a disposable micropipet and has the dimensions 1.3 mm i.d. × 32 mm long, with an internal volume of about 30 μl. It holds about 10 mg of porous glass beads. As described under Methods, the ends of the capillary can be sealed using either a sintered glass plug or a porous plug made by precipitating polyvinidylene difluoride in the interstices between the beads. The latter method is simpler and more suitable for bulk processing, but occasionally imperfect plugs are made, so it is necessary to test each column by forcing through liquid and observing whether any beads are lost. After packing, the column should be washed and dried thoroughly to prevent decomposition of the support.

Application of the sample to the column is done by capillary action. The dissolved sample is placed in a small plastic tube (e.g., an Eppendorf tube), one end of the capillary column is dipped into the solution, and the liquid is drawn into the capillary (Fig. 1A). The volume of the sample solution is chosen such that it fills about one-half of the capillary. Then the sample tube is washed with another volume of solvent. In this way, nearly 98% of the sample can be applied to the column.

For sequencing, the capillary column is placed in a column holder as shown in Fig. 1B. The capillary is inserted into the flared ends of two lengths of 0.012-in.-i.d. Teflon tubing which are connected to the sequencer. The capil-

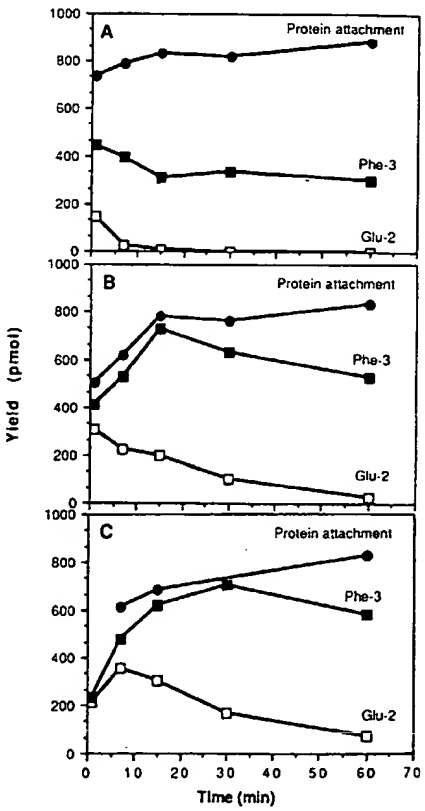

FIG. 6. Effect of time, temperature, and EDC concentration on attachment yield and sequencing results for *S. cerevisiae* cytochrome c. Protein samples (1000 pmol each, in 15 μl of buffer) were activated under the following conditions: (A) room temperature, 2 mg EDC; (B) 0°C, 2 mg EDC; (C) 0°C, 1 mg EDC. Attachment yields were calculated by measurement of radioactivity. Yields of PTH-Glu-2 and PTH-Phe-3 were determined by HPLC analysis after sequencing.

TABLE 2

Attachment and Sequencing Yields of Proteins and Peptides in Arylamine-Glass Capillary Columns[a]

Protein or peptide	Initial amount (pmol)	Attachment yield (%)	Sequenceable amount[b] (pmol)	Overall sequenceable yield[c] (%)
β-Lactoglobulin	100	76[d]	—	—
	100	85[e]	37	37
Myoglobin	100	81[d]	—	—
	100	86[e]	48	48
Glyceraldehyde-3-phosphate dehydrogenase	100	77[d]	—	—
Bovine serum albumin	100	87[d]	—	—
Trypsinogen	100	85[d]	—	—
Cytochrome c	100	75[e]	56	56
Ribonuclease a	100	77[e]	42	42
SAPYTYQSDLRYKL	80	—	51	64
WDYHNRMK	80	—	24	30
SHEGTFTSDYSKYLDSRRAQSFVQWL	110	—	80	70
DPIDGRVIGS	72	—	32	44
GIVEQCCASVCSLYQLENYCN	85	—	36	42
GHEALTGTEKLIETYFNH	102	—	34	33

[a] Samples were coupled in arylamine-glass capillary columns at 0°C for 15 min using 1 mg of EDC.

[b] The amount of attached peptide capable of being sequenced was determined by integrating the areas of the first three PTH peaks and extrapolating to zero cycles.

[c] The overall yield reflects both the attachment and sequenceable yields.

[d] Attachment yields for ^{125}I-labeled proteins. Yields were calculated based on the combined radioactivity of the columns and washings. Duplicate samples were run, giving yields within 5% of each other.

[e] Yield determined by amino acid analysis. Duplicate samples were prepared; one was used for amino acid analysis and the other for sequencing.

lary and Teflon tubes are secured in an aluminum column holder using two tube end fittings (Rainin Instruments). The column holder can be constructed with a variety of external dimensions to conform to those of the sample compartment of the particular model of sequencer.

DITC-glass capillary columns. The efficiency of attachment of proteins to DITC-glass was determined by coupling ^{125}I-labeled proteins to the support. Figure 2 shows that attachment is essentially complete in about 20 min at 55°C, pH 9.0. The attachment buffer (0.2 M sodium phosphate, pH 9.0, 1% SDS) is suitable for dissolving most proteins. Other buffers can be used, but it is important that buffers (and the protein sample) not contain primary or secondary amines, thiols, or other substances which may react with isothiocyanate groups. Excess isothiocyanate groups on the support are blocked in the first cycle of Edman degradation by reaction with 0.1% cyclohexylamine, which is present in the phenyl isothiocyanate coupling buffer.

To determine the distribution of the protein in the capillary column, a column containing a radioiodinated protein was cut into four sections, which were counted. Approximately 75% of the protein was concentrated in the first quarter of the capillary, with most of the remainder being in the second quarter. Since the column filling time is only a few seconds, and the attachment reaction half-life is several minutes (Fig. 2), the concentration of the protein is probably due to binding to the hydrophobic DITC groups. The capacity of the DITC-glass capillary column is >50 nmol for most proteins, or a 500-fold excess over the amount of protein used in these experiments.

As can be seen in Table 1, the attachment efficiencies range from about 80 to 92%, which is comparable to yields using earlier methods. The less than quantitative results are not due to inefficient transfer of the sample into the capillary, however, since counting of the empty Eppendorf tube and the capillary column shows that 98% of the protein entered the capillary. A possible concern is that protein samples evaporated in Eppendorf tubes may not redissolve completely. This did not seem to be a problem with any of the proteins used in these studies, since all of the attachment buffers contain 0.1% SDS. However, the potential problem can be avoided by adding SDS to the sample *before* evaporating it. Sequencing of the immobilized proteins revealed that only about half of the attached material was available for Edman degradation. The cause of this loss is not known, but these results compare favorably with initial yields of 20–55% seen for proteins blotted onto PVDF membranes and sequenced by the gas-phase method (13).

Figure 3 shows the results of sequencing *Saccharomyces cerevisiae* cytochrome c on a DITC-glass column.

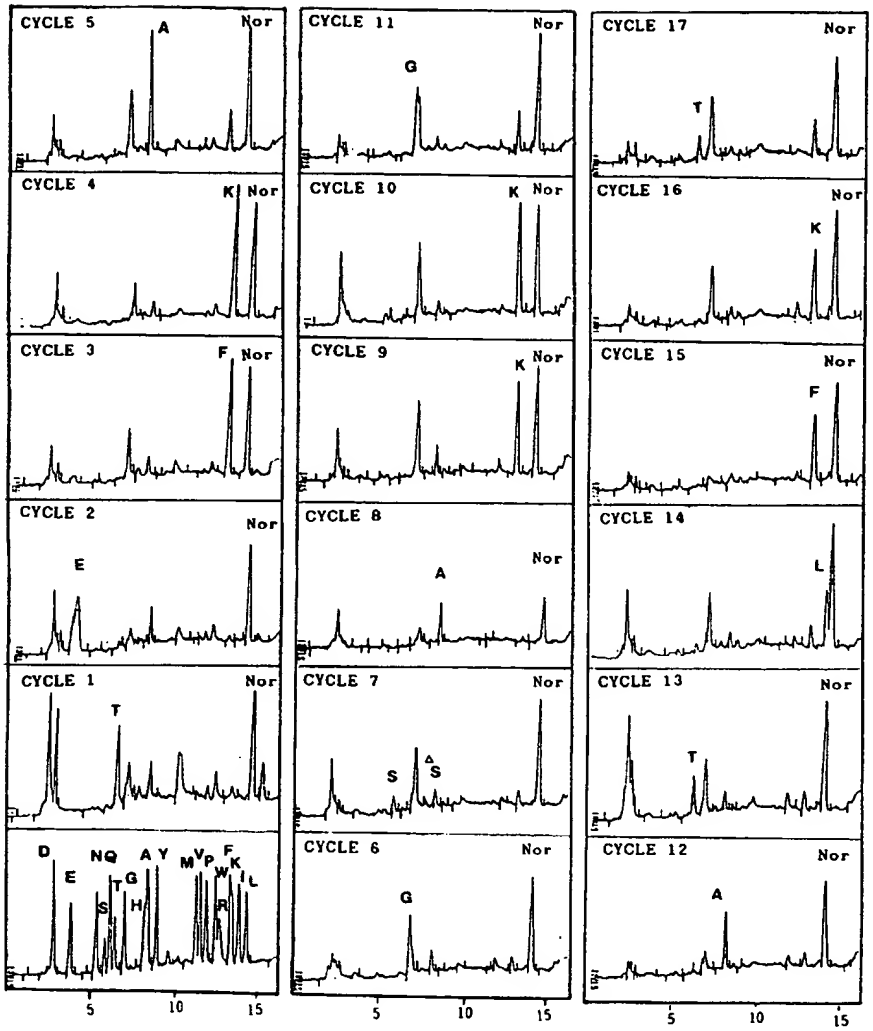

FIG. 7. HPLC traces of PTH-amino acids from sequencing of *S. cerevisiae* cytochrome c coupled to AP-glass in a capillary column. A 100-pmol sample was coupled as described in the text at 0°C for 15 min with 1 mg of EDC before sequencing. The PTH standard is shown in the lower left panel. The norleucine internal standard (20 pmol) appears at about 14 min and a reaction by-product, aniline, at 7 min.

Note that the N-terminal amino acid and some of the lysine residues are recovered in low or negligible yield because of their being covalently attached to the support.

Aminophenyl-glass capillary columns. Arylamine resins (14) and glass (15) supports were introduced on the premise that an arylamine, which is only half protonated at pH 5, would be a much better nucleophile than a more basic alkylamine support in carbodiimide-mediated immobilization of proteins via their carboxyl groups. In fact, attachment of proteins to AP-glass is extremely fast, being nearly complete at room temperature, using 1–2 mg of EDC per sample, in 1–2 min (Fig. 4). The rapid reaction was confirmed by analysis of the distribution of attached protein as described above for DITC-glass, which showed that >95% of the protein is concentrated in the first quarter of the capillary column.

The reaction is carried out by adding a freshly made solution of EDC to the protein sample and immediately drawing the reaction mixture into the capillary column. As seen in Fig. 5, if one waits, after activation of the protein with EDC, before applying it to the AP-glass, the attachment yield decreases significantly, presumably because of rearrangement of the *O*-acylurea active intermediate to the *N*-acyl urea. It is also important that the sample not contain carboxylic substances, such as acetate, which would compete with protein carboxyls for sites on the AP-glass support.

Initial experiments showed that a substantial percentage of side-chain carboxyl groups become attached to the AP-glass support, resulting in low yields of PTH-Asp and PTH-Glu on sequencing. Figure 6A shows that at room temperature with 2 mg of EDC per sample, the attachment was complete within a few minutes, but that

the amount of sequenceable protein was only about 30%, as indicated by the yields of PTH-Phe at position 3, and that no PTH-Glu was seen at position 2 because its side-chain carboxyl was coupled to the support. By lowering the reaction temperature to 0°C, the attachment reaction was slowed down, and both the sequenceable yield and the yield of PTH-Glu were improved (Fig. 6B). A further improvement was made by reducing the EDC concentration to 1 mg per sample (Fig. 6C). Based on these experiments, we chose as standard reaction conditions, 0°C, 1 mg per sample of EDC, and 15 min, which represents a compromise between high attachment yield, good sequenceability, and acceptable recoveries of PTH-Glu and PTH-Asp. If one allows the reaction to proceed too long, the carboxyls couple too efficiently to the support and apparently also to the N terminus of the protein, blocking it to Edman degradation.

As can be seen in Table 2, attachment and sequenceable yields for AP-glass columns are similar to those found for DITC-glass. Figures 7 and 3B show the results of sequencing cytochrome c on AP-glass. In contrast with DITC-glass (Fig. 3A) all of the amino acid PTHs are recovered in good yield, including PTH-Glu. It is also noteworthy that the repetitive yield is higher than for DITC-glass, primarily because of reduced carryover. AP-glass columns are particularly useful for immobilization of peptides, since the majority of peptides do not contain C-terminal lysines that allow them to be attached to DITC-glass.

AEAP-glass capillary columns. As described earlier (12), AEAP supports are useful for selective coupling of homoserine lactone peptides generated by cleavage at methionine with cyanogen bromide. AEAP-glass capillary columns are also effective for immobilizing such peptides. Attachment of 220 pmol of the β-lactoglobulin cyanogen bromide peptide, KGLDIQKVAGTWYSLAM, resulted in 143 pmol (65%) of sequenceable peptide with a repetitive yield of 93%, the latter being slightly lower than for other methods because of the acid lability of homoserine peptide bonds.

SUMMARY

The capillary column attachment method produces results in terms of efficiency of immobilization and protein sequencability at least comparable to those of membrane adsorption (13), DITC-glass fiber (2), and SequeNet (unpublished results of MilliGen/BioSearch Division of Millipore Corp.) methodologies. This method is particularly useful for covalent immobilization of peptides at their carboxyl-terminal ends, using carbodiimide coupling. The capillary columns described are useful for samples ranging in amount from about 10 pmol to 50 nmol. This is probably due to the large surface area of the glass beads and the large excess of polypeptide binding sites. The much higher capacity of the capillary columns, compared to membrane supports, is of advantage for extended run sequencing, where larger protein samples are needed and for analysis of synthetic peptides for failure sequences. Finally, this technique permits the efficient direct transfer of proteins from polyacrylamide gels by electroelution from gel slices onto DITC-glass capillary columns (to be published).

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